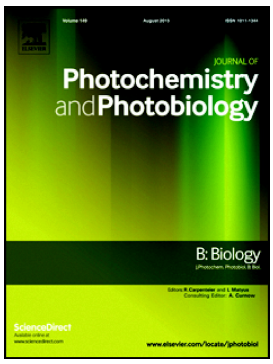

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Bioluminescent and structural features of native folded Gaussia luciferase

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Abstract

The secreted luciferases responsible for light emission of marine copepods have gained popularity for being used in noninvasive imaging of intracellular events. The secreted luciferase of copepod *Gaussia princeps* is a one-subunit protein catalyzing coelenterazine oxidation to emit blue light. It consists of the *N*-terminal variable part that bears a signal peptide for secretion and the *C*-terminal catalytic domain containing ten highly conserved Cys residues supposing the existence of up to five S-S bonds. Despite wide application of Gaussia luciferase in biomedical research, its biochemical properties are still insufficiently studied due to the general problem of obtaining the proper folded Cys-rich proteins in bacterial cells. Here we report the properties of the proper folded Gaussia luciferase produced in insect cells using baculovirus expression system. This high purity luciferase reveals the highest activity at 15-20 ^{o}C but retains only ~20% activity at 37 ^{o}C that may hamper its application for *in vivo* assays. The maximum of bioluminescent activity of GpLuc is found at NaCl concentrations in the range of 1.0-1.5 M and, furthermore, a high NaCl concentration enhances luciferase stability to thermal denaturation, i.e. Gaussia luciferase displays the features characteristic of halophilic enzymes. The studies on bioluminescence kinetics at different coelenterazine concentrations obviously show a positive cooperativity of Gaussia luciferase with coelenterazine (Hill coefficient – 1.8 ± 0.2; $K_{0.5}$ – 2.14 ± 0.17 μM). We suggest this effect to be rather due to the so-called kinetic cooperativity conditioned by conformational changes in response to substrate binding than to the presence of two catalytic sites.

1. Introduction

Continuous noninvasive imaging of intracellular events provides important information on cell physiology. For these purposes, a variety of bioluminescent and fluorescent proteins originating from marine luminous and nonluminous organisms are widely and successfully applied as genetically encoded reporters [1,2]. Nowadays firefly, bacterial, and Renilla luciferases as well as Ca^{2+}-regulated photoproteins are the most popular bioluminescent reporters that are used for monitoring gene expression, tracing of labeled cells in small experimental animals, estimation of cell viability, apoptosis, and various processes associated with cellular metabolism, measurement of intracellular calcium transients in response to different stimuli etc. However, in the last decade, application of the naturally occurring secreted luciferases has gained popularity. The excellence of secreted luciferases over the other bioluminescent reporters is that they allow extracellular recording of intracellular events without destroying cells or tissues.

The secreted luciferases are responsible for the light emission of a variety of organisms, mostly crustaceans [3]. The cDNAs encoding the luciferases from ostracods *Vargula hilgendorfii* [4] and *Cypridina noctiluca* [5], shrimp *Oplophorus gracilorostris* [6] and several different copepod species [7-11] have been cloned. Although all these luciferases have been expressed in mammalian cells and all of them have shown promising characteristics as secreted reporters, the luciferases from copepods *Gaussia princeps* and *Metridia longa* are the only ones widely used in biomedical research and in the development of cell-based assays for high throughput screening [12,13].

Bioluminescence of the marine copepod *Gaussia princeps* is conditioned by a secreted one-subunit luciferase with a molecular mass of 19.9 kDa [8]. The Gaussia luciferase (GpLuc) catalyzes the oxidative decarboxylation of coelenterazine molecule in the course of which an electronically excited product, coelenteramide, is formed. Its relaxation to the

ground state generates blue light. Similar to other copepod luciferases, the GpLuc consists of the *N*-terminal variable part bearing a signal peptide for secretion and the *C*-terminal catalytic domain containing ten highly conserved Cys residues supposing the existence of up to five S-S bonds per luciferase molecule (Fig. 1) [12].

Since GpLuc is widely applied as a reporter in biomedical research *in vivo*, the attempts to characterize its biochemical and bioluminescent properties have been repeatedly undertaken. The main hindrance to properly determine enzymatic characteristics of GpLuc is a production of the correctly folded high purity protein. Primarily, it is due to the difficulties of obtaining a functionally active luciferase in bacterial cells. The prokaryotic and eukaryotic cells maintain reductive environment in cytoplasm that hampers the formation of disulfide bonds and consequently the proper folding of Cys-rich proteins. Several approaches have been used to overcome this obstacle. For instance, GpLuc was expressed in *Escherichia coli* periplasm using a specific pelB signal peptide for secretion [15]. The *E. coli* periplasm is rightly considered to be suitable for expression of proteins comprising S-S bonds because this compartment contains disulfide oxidoreductase and disulfide isomerase—the enzymes that catalyze the formation of disulfide bonds [16]. Of note is that it might be inefficient for Cys-rich proteins [16] which GpLuc belongs to. The other tested approaches aimed at enhancing GpLuc solubility in *E. coli*. These included lowering of the expression temperature (pCold expression system) [17] and expression of GpLuc as a fusion with synthetic IgG-binding domain of the protein A [18], solubility enhancement peptide [19], or *C*-terminal 15-amino acid Tyr linker [20]. In addition, there were attempts to produce GpLuc by refolding from inclusion bodies [21] and by synthesis in a cell-free system [22]. Although these approaches allow the production of Cys-rich proteins, none of them guarantees the correspondence of the properties of the recombinant protein obtained to those of the native protein until they are compared as it was made, e.g. for the Metridia luciferase isoform refolded from inclusion

bodies [23].

The secreted expression in eukaryotic cells such as insect cells with the use of baculovirus expression system allows the production of Cys-rich proteins like copepod luciferases with a proper pattern of disulfide bonds. Thus, the properties of the proteins produced only in eukaryotic cells will fully correlate with those of luciferases used as reporters in mammalian cells and small animals. Although many genes for copepod luciferases have been identified [12], the thorough characterization of native folded recombinant proteins has been performed only for MLuc7 and MLuc2 isoforms of *Metridia longa* luciferase (Fig. 1) [10,11].

In the present study, we report the expression of Gaussia luciferase in insect cells, its purification from culture medium and characterization of a high purity protein.

2. Materials and Methods

2.1. Materials

Coelenterazine was obtained from NanoLight Technology, a division of Prolume Ltd. (Pinetop, AZ, USA). Its concentration was calculated by absorption at 435 nm using the $\varepsilon_{435\ nm}$ = 9800 $cm^{-1}M^{-1}$ [24]. All other chemicals were obtained from Sigma-Aldrich and used without further purification unless otherwise stated.

The plasmid bearing the gene encoding GpLuc was a gift of Bruce Bryan, MD at NanoLight.com.

2.2. Production of Gaussia Luciferase in Insect Sf9 Cells and Its Purification

For secreted expression in insect Sf9 cells, the Bac-to-Bac Baculovirus expression system (Invitrogen) was used. The GpLuc coding sequence with native signal peptide was amplified using pCMV-GLuc template (NanoLight Technology) with humanized Gaussia luciferase and specific primers: forward 5'-ACAGGTACCATGGGAGTCAAAGTTCTG-3' with KpnI site (underlined) and two overlapping reverse primers: first 5'-TGATGATGACCTTGAAAGTACAAGTTCTCGTCACCACCGGCCCCC-3' and second

5'-TACTCGAGTCATTAGTGATGGTGATGGTGATGATGACCTTGAAAG-3' with XhoI site by two-step PCR technique as described for MLuc164 [7]. The oligonucleotide primers were designed to introduce the C-terminal His7-tag followed by a TEV-specific protease site after GpLuc coding sequence. After digestion, the synthesized fragment encoding GpLuc-His7 was cloned into KpnI/XhoI sites of the modified pFastBac1 expression plasmid (Invitrogen), in which BamHI site was turned into KpnI site. Generation of the recombinant bacmid DNA using DH10Bac *E. coli* cells, transfection of Sf9 cells with recombinant bacmid-GpLuc, obtaining of amplified recombinant GpLuc-baculovirus, and virus titration were done according to manufacturer's manual for the Bac-to-Bac Baculovirus expression system. The GpLuc bioluminescence activity was used to evaluate the infection efficiency.

The secreted expression of GpLuc in insect cells and its purification from culture medium were performed as it was previously reported for isoform MLuc2 of Metridia luciferase [11]. The Sf9 cells (Invitrogen) cultured in suspension at 28 ^{o}C without CO_2 using serum-free medium Sf900 II SFM (Life Technologies) were infected with the P2 viral stock (titer ~5 × 10^7 infectious units per 1 mL) at a multiplicity of infection (MOI) of 2 plaque-forming units and harvested at 72 h post-infection. Cells were pelleted at 2000g for 10 min at 4 ^{o}C and GpLuc was immediately concentrated from culture medium by differential precipitation (40–65%, w/v) with $(NH_4)_2SO_4$. Then, the insoluble particles were spun down (6000g, 20 min), dissolved in Ni-binding buffer (0.15 M NaCl, 30 mM imidazole, 20 mM Tris-HCl, pH 7.5), and passed over a 5-mL HisTrap column (GE Healthcare). The luciferase was eluted with 0.3 M imidazole in the same buffer. The luciferase peak was concentrated with Amicon Ultra Centrifugal Filter (EMD Millipore) and the elution buffer was changed for TEV protease cleavage buffer (0.15 M NaCl, 1 mM EDTA, 0.02 mM DTT, 20 mM Tris-HCl, pH 7.5). The His6-tag was digested with TEV protease at the ratio 50:1 (w/w) by overnight incubation of the luciferase sample at 4 ^{o}C. Then the sample was loaded on a Ni-NTA column and the flow-

through was collected. The collected sample was concentrated with the replacement of imidazole-containing buffer to the buffer 0.15 M NaCl, 5% glycerol, 0.02% NP-40, 20 mM Tris-HCl pH 7.5 (storage buffer) and passed through a Superdex75 gel filtration column (GE Healthcare) equilibrated with the same buffer. After this chromatography step, GpLuc was of a high purity (Fig. 2). The yield of GpLuc luciferase was ~6 mg/L of insect cell culture. The monomericity of GpLuc was confirmed by non-reducing PAGE and gel filtration on Superdex-75 column. For storage, the luciferase was transferred into buffer 0.15 M NaCl, 1 mM EDTA, 0.02% NP-40, 20 mM Tris-HCl, pH 7.5. For long storage, the sample was supplemented with 50% glycerol and stored at -20 ^{o}C. Under these conditions, GpLuc completely retains activity for several months.

Protein concentration was determined using a DC^{TM} Protein Assay kit (Bio-Rad). Free thiol groups were determined with the Ellman's reagent (Sigma) according to manufacturer's directions.

2.3. Bioluminescence Assay and Spectral Measurements

Bioluminescence was measured by rapid injection of 5 μL of coelenterazine methanol solution into a luminometer cell containing a protein sample in 0.5 mL of the buffer (ML) 0.5 M NaCl, 0.015% gelatine, 50 mM Tris-HCl pH 7.5. This assay buffer was used in all measurements of bioluminescent activity, unless otherwise stated. The temperature of the assay tube was supported with a temperature Peltier-controlled cell holder. The luciferase activity was estimated by the maximal luminescent signal.

The bioluminescence and fluorescence spectra were measured with a Cary Eclipse spectrofluorimeter (Agilent Technologies, USA) equipped with a temperature Peltier-controlled cell holder. The obtained spectra were corrected for spectral sensitivity of the instrument. The bioluminescence was initiated by injection of coelenterazine in methanol solution (enzyme/substrate molar ratio was ~1:1000). The bioluminescence spectra were

measured in the range of 400–600 nm at a slit width of 5 nm and with spectral scanning speed of 400 nm/s.

2.4. Thermal Unfolding

Thermally induced luciferase unfolding was detected by measuring Trp fluorescence [25] (λ_{ex} = 295 nm, slit width 5 nm) at a fixed wavelength of 330 nm by gradually increasing the temperature in the protein samples from 20 to 95 ^{o}C with a heating rate of 1 ^{o}C/min. The values of completeness of transition α were calculated according to the equation (1):

$$\alpha = (I - I_f)/(I_u - I_f) \quad (1)$$

where I_f and I_u parameters characterize the intensities of Trp fluorescence of folded and unfolded protein fractions, respectively. The final curves were normalized and fitted with the Boltzman sigmoidal function in OriginPro 9.0 software (OriginLab Corp., Northampton, MA) that describes the transition of normalized parameter from 0 to 1 as temperature (T) grows:

$$P_{norm}(T) = 1 - 1/[1 + e^{\frac{(T-T_{0.5})}{d}}] \quad (2)$$

where thermal transition midpoint $T_m = T_{0.5}$. The measurements were performed in 20 mM HEPES pH 7.5 containing either 0.15 or 1.0 M NaCl. Protein concentration was 0.1-0.15 mg/mL.

2.5. Rapid-mixing Kinetics

The kinetics of light response at fast mixing of luciferase sample and coelenterazine solution was examined using an Applied Photophysics SX20 stopped-flow machine (20 μL cell volume, 1.1 ms deadtime). The temperature was controlled using a circulating water bath and was set at 18 ^{o}C. The syringes contained a protein sample of concentration 50 nM in 150 mM NaCl, 20 mM Tris-HCl pH 7.5 and various concentrations of coelenterazine freshly prepared in 150 mM NaCl, 20 mM Tris-HCl pH 7.5, respectively. The solutions were mixed in equal volumes.

3. Results and Discussion

3.1. Biochemical and Bioluminescent Properties

The bioluminescence of marine copepods originates in response to different stimuli as a secretion of a luminous bolus in sea water [12]. It implies that copepod luciferases have evolved to function in the conditions characteristic of sea water environment (salinity, temperature, pH etc.). In particular, one of the Metridia luciferase isoforms is an extremely psychrophilic enzyme with a temperature optimum at 5 ^{o}C [11] whereas the other one displays bioluminescence activity at 12-17 ^{o}C [10]. It may exemplify adaptation of bioluminescence of copepod *Metridia longa* to the changes in water temperature during diurnal vertical migrations from the depth where the temperature is near zero to the surface where it can reach 24 ^{o}C.

Figure 3 summarizes the effect of temperature, pH, and salt concentration on light intensity of a high purity GpLuc purified from culture medium of insect cells. The GpLuc displays a temperature optimum (T_{opt}) of bioluminescence at ~15-20 ^{o}C (Fig. 2A) that almost matches that of MLuc7 isoform of Metridia luciferase [10]. The GpLuc retains ~30% activity at 1 ^{o}C but only ~20% activity at 37 ^{o}C. Thus, a substantial decrease of activity at 37 ^{o}C may hamper the application of GpLuc as a reporter for *in vivo* assays. Noteworthy, although the temperature optimum of GpLuc bioluminescence is close to that of MLuc7 (12-17 ^{o}C), GpLuc shares greater sequence identity (Fig. 1) with a psychrophilic isoform of Metridia luciferase which T_{opt} is ~5 ^{o}C [11]. The pH optimum of GpLuc bioluminescence is found in the narrow range of 7.5-8.5 (Fig. 3B) that lies in the same pH range as for the isoforms of Metridia luciferase [10,11,26]. However, GpLuc is more active under alkaline pH than Metridia luciferase isoforms; GpLuc displays ~60% activity at pH 9.0 whereas MLuc isoforms retain only ~20%. Of note is that the bioluminescence pH optima of both GpLuc and MLuc isoforms are in the range of pH characteristic of sea water (7.5-8.4). The effect of salt

concentration on light intensity of GpLuc is shown in Figure 3C. In contrast to Metridia luciferase, isoforms MLuc7 and MLuc164 with the highest activity at 0.5 M [10,26] which is close to NaCl concentration in sea water, GpLuc reveals the maximum of bioluminescent activity at NaCl concentrations in the range of 1.0-1.5 M, i.e. practically at concentrations as for psychrophilic isoform MLuc2 of Metridia luciferase [11]. The requirement of high salt concentration for catalytic activity is a feature characteristic of halophilic enzymes [27]. The halophilic enzymes are frequently inactivated at low salt concentrations. Among copepod luciferases studied, MLuc7 [10] and GpLuc only (Fig. 3C) retain a substantial bioluminescent activity (~50 and 20%, respectively) in the absence of salt. Although the isoforms MLuc2 and MLuc164 of Metridia luciferase differ in optimal salt concentrations for bioluminescence, both of them are virtually inactive in salt-free solutions [11,26]. Of note is that the substitution of NaCl by KCl does not significantly alter the dependence of light intensity on salt concentrations (Fig. 3C).

The high purity GpLuc produced in insect cells reveals an extremely high thermostability (Fig. 3D) similar to other copepod luciferases [9-11]; GpLuc retains up to 35% of the initial activity even after 2-h exposure at 100 ^{o}C. The effective renaturation of these luciferases after heating is most likely due to the presence of intramolecular disulfide bonds. Actually, no free SH-groups (0.2 ± 0.1, n = 3) were found in GpLuc at denaturing conditions, i.e. GpLuc like MLuc7 [10] also contains five intramolecular S-S bonds per molecule.

The GpLuc purified from culture medium of insect cells displays a light emission peak with λ_{max} at 485 nm (Fig. 4A) that matches those of other copepod luciferases [7,9-11,26]. This is not surprising because the *C*-terminal domains of copepod luciferases where the active site is located [14] are highly conserved (Fig. 1C). For instance, the degree of identity of amino acid sequences of hydromedusan Ca^{2+}-regulated photoproteins isolated from different organisms amounts to ~60-70% [28] which is even less than that for the conserved *C*-terminal

domains of copepod luciferases (Fig. 1C). At the same time, the residues that form the photoprotein substrate-binding cavity and interact with 2-hydroperoxycoelenterazine molecule are practically identical in all hydromedusan photoproteins [29]. Even the difference in one amino acid affects the bioluminescence spectrum of the photoprotein [30,31]. Thus, we can reasonably suppose that the key amino acid residues involved in the oxidative decarboxylation of coelenterazine molecule and the emitter formation are to be identical at least in Metridia and Gaussia luciferases.

Of note is that the change of pH in the range 6.0-9.0 does not influence the light emission spectrum of Gaussia luciferase (Fig. 4A) in contrast to firefly-type luciferases for which the change of pH from 8.0 to 6.0 may shift the bioluminescence spectrum maximum for almost 100 nm [32,33].

The dependence of GpLuc light intensity on protein concentration is linear over a wide range of luciferase concentrations (Fig. 4B) as it is observed for Metridia luciferase isoforms produced in insect cells [10,11]. With an ordinary luminometer applied, the GpLuc can be detected at concentration of 0.46 fM, i.e. GpLuc detection limit exactly corresponds to that of MLuc7 isoform (0.43 fM [10]) of Metridia luciferase. This evidently shows that GpLuc and MLuc7 have the same specific bioluminescence activities and consequently they must provide equal sensitivities of *in vivo* and *in vitro* assays.

3.2. Structural Features

In contrast to other coelenterazine-dependent bioluminescent proteins [12,29], copepod luciferases are very poor in Tyr and Trp residues—GpLuc has only one tryptophan and one tyrosine, while Metridia luciferase isoforms contain one additional Tyr instead of Phe in GpLuc (Fig. 1). Recently it was demonstrated that Tyr residues may be involved in the formation of an active site of copepod luciferases [34]. Due to a low content of Tyr and Trp residues, the intrinsic fluorescence of copepod luciferases is rather weak. Like the Metridia

luciferase isoforms [11,34], with excitation at 275 and 295 nm GpLuc fluoresces with the maxima at 303 and 330 nm characteristic of Tyr and Trp fluorescence (data not shown), respectively. The intensity of Tyr fluorescence in the case of GpLuc also exceeds that conditioned by Trp residue [11,34]. It obviously demonstrates that the environments of the side chains of aromatic residues in GpLuc and MLuc have to be identical and consequently their spatial structures should be very similar or the same.

The thermal unfolding of GpLuc monitored by the change in Trp fluorescence at 330 nm is shown in Figure 5. The melting temperature (T_m) calculated from denaturation curve amounts to 72.1 ± 0.6 ºC. This value practically corresponds to T_m determined for MLuc7 isoform of Metridia luciferase [11,23]. The high T_m values indicate that the protein molecules of both luciferases have a high structural rigidity most likely owing to the presence of five disulfide bonds. NaCl at concentration of 1 M further enhances the stability of GpLuc (T_m = 77.3 ± 0.7 ºC). This property makes GpLuc resemble halophilic enzymes which resistance to denaturation goes up as salt concentration increases [27,35].

Noteworthy is that the melting temperature we determined for GpLuc produced from culture medium of insect cells differs from that (T_m is ~60 ^{o}C [19]) calculated from changes of CD spectra for GpLuc produced in *E. coli* cells. It suggests that the conformations of GpLuc produced in *E. coli* and insect cells may differ despite the fact that the luciferase from bacterial cells was active [19].

3.3. Rapid-mixing Bioluminescence Kinetics of GpLuc

The dependence of bioluminescence of GpLuc produced in *E. coli* cells with regard to coelenterazine concentration has revealed a positive cooperativity [36]. For high purity GpLuc purified from culture medium of insect cells, we have also examined the dependence of initial light intensity on a substrate concentration in order to be sure that the cooperativity phenomenon is not due to the technique the protein was produced by. Indeed, light intensity

of GpLuc produced by insect cells responses nonlinearly to the change of coelenterazine concentration (Fig. 6). The Hill coefficient (h) calculated from the curve slope in Figure 6 (insert) is equal to 1.8 ± 0.2. Although the value we have determined is 1.5-fold less than that found for GpLuc from *E. coli* cells (h = 2.9 [36]), the Hill coefficient of 1.8 is sufficient to describe the positive cooperativity of GpLuc for substrate. The midpoint of GpLuc kinetic response ($K_{0.5}$) equals 2.14 ± 0.17 μM.

In addition, we have studied fast bioluminescence kinetics of GpLuc at different coelenterazine concentrations (Table 1, Fig. 7). Similar to the initial light intensity, the rise (k_{rise}) and decay (k_{d1}) rates of bioluminescent signal are nonlinearly growing with the increase of coelenterazine concentration. Of note is that when the substrate concentration exceeds that of luciferase 100-fold, the decay kinetics can be satisfactorily described only by a two-exponential decay function and consequently by two rate constants—"fast" (k_{d1}) and "slow" (k_{d2}) (Table 1).

The copepod luciferases comprise two repeat sequences (Fig. 1B) which most likely arise from duplication of one precursor gene [7]. It was reported [21] that the repeats display a very weak bioluminescence activity when separately expressed in *E. coli* cells. It allowed suggesting two active sites in copepod luciferases. However, another study showed [37] the GpLuc repeats to reveal no activity at their separated expression in mammalian cells. Of note is that we also expressed the repeats, but only those belonging to Metridia luciferase, in *E. coli* cells and found no bioluminescent activity in any of them. Since presently the bioluminescent activity of individual repeats remains questionable, we may hardly consider two catalytic sites as a reason of positive cooperativity. The case we have come across with copepod luciferases is most likely the so-called kinetic cooperativity [38]. There are monomeric enzymes containing single substrate-binding site but displaying cooperativity. The cooperativity arises as a result of conformational changes in response to ligand binding

occurring on a timescale comparable to the catalytic rate constant [38]. The best studied representative of this type of enzymes is a human glucokinase. This enzyme has single binding site for glucose but displays a cooperativity kinetic response to substrate characterized by a Hill coefficient of 1.7 [39,40]. According to NMR studies [40], the intrinsic disordered domain of glucokinase undergoes substantial rearrangement upon glucose binding that, in fact, is a cause of the positive cooperativity.

With regard to the predicted secondary structure (Fig. S1), GpLuc is poor in α-helices and β-strands but contains the extended coil regions which are found even in the *C*-terminal domain where the luciferase active site is located [14,34]. Furthermore, the intrinsic disordered regions in a catalytically important part of luciferase may also be present. Thus, the speculation that positive cooperativity in copepod luciferases is rather conditioned by kinetic cooperativity than by the existence of two catalytic sites looks quite plausible. This assumption is also supported by the fact that the greatest bioluminescence activity is observed at high salt concentrations (Fig. 2C) providing higher structural rigidity of GpLuc molecule.

3.4. Comparison of Properties of GpLuc Purified from the Culture Medium of Insect and CHO Cells

When the manuscript describing the results of this work was under review, an independent study on the secreted expression of GpLuc in CHO cells, GpLuc purification from culture medium, and some characterization of high purity enzyme was reported [41]. A brief comparison of our GpLuc sample and the one obtained in CHO cells [41] will be appended here. Both luciferases reveal the same spectral maxima of light emission (Fig. 4A), bioluminescence pH optima (Fig. 3B), and a wide linear range of the dependence of light intensity on protein concentration (Fig. 4B). Although the bioluminescence kinetics of GpLuc produced in CHO cells was not thoroughly studied [41], the author mentions that the decay rate of light signal grows as coelenterazine concentration increases, i.e. similar to what was

found in our studies (Table 1). However, one significant distinction is that GpLuc produced in insect cells reveals the highest activity at salt concentration of 1.0-1.5 M (Fig. 3C), whereas the optimal NaCl concentration for light emission of GpLuc from CHO cells is only 0.15 M [41]. This difference may be accounted for by the presence of His-tag in GpLuc from CHO cells at C-terminus (in our sample His-tag was removed). The determined K_m can hardly characterize GpLuc properly [41] since GpLuc displays positive cooperativity with a substrate and consequently it is not likely that Michaelis-Menten equation can be applied to describe it [38].

4. Conclusion

In summary, the present work reports the bioluminescent properties and structural features of the native folded high purity Gaussia luciferase produced by secreted expression in insect cells. We have found that the highest bioluminescent activity of GpLuc is observed at 15-20 ^oC and the luciferase retains only 20% of its activity at 37 ^oC. Furthermore, we have established that the optimal salt concentration for GpLuc functioning lies in the range of 1-1.5 M and enhances GpLuc stability to thermal denaturation (T_m = 72.1 ± 0.6ºC at 0.15 M *vs.* T_m = 77.3 ± 0.7 ºC at 1.0 M), i.e., in fact, Gaussia luciferase displays the peculiar features of halophilic enzymes [27,35]. In addition, we have confirmed that GpLuc shows positive cooperativity with coelenterazine as it was earlier described for copepod luciferases produced in *E. coli* cells [36]. However, we suppose that this effect arises owing rather to the so-called kinetic cooperativity [38] conditioned by the conformational changes in response to substrate binding than to the existence of two catalytic sites [21,36]. It is worth noting that only determination of spatial structure of copepod luciferase might shed light on the mechanism of cooperativity. Thus, the properties of GpLuc determined in this study are highly supposed to correspond to those of GpLuc at its use as a reporter in the assays *in vivo*. This makes us hope that our results may help the researchers in designing the experiments and proper

interpretation of the obtained data.

Bioluminescent and biochemical properties of Renilla, Cypridina, and Oplophorus luciferases that also use the imidazopyrazinone-type luciferin were first studied with regard to natural enzymes isolated from the corresponding luminous organisms [12]. As opposed to the luciferases mentioned, the natural luciferases of copepods have not been characterized yet. The natural intracellular Renilla [42] and secreted Cypridina [43] luciferases were isolated as monomeric proteins. At the same time, the secreted luciferase of the decapod shrimp *Oplophorus gracilirostris* was purified as a tetrameric protein composed of two monomers, each of M_r = 19 kDa and two monomers, each of M_r = 35 kDa [44], but only the low molecular mass monomers revealed the luciferase activity. The function of other subunits still remains unclear. The secreted bioluminescence of copepod arises after injection of luciferase and substrate into sea water. However, coelenterazine is unstable in aquatic solutions in the presence of molecular oxygen [24]. Additionally, as coelenterazine can easily permeate the membranes of eukaryotic cells [45] (an important feature for the development of *in vivo* assays [12]), it would be distributed throughout the whole organism unless there is "trick" to hold the coelenterazine before secretion begins. Bioluminescence of the soft coral Renilla, for example, involves Ca^{2+}-regulated coelenterazine-binding protein (CBP) [46] carrying out a function of stabilizing coelenterazine [47,48] and linking with nervous system through the changes of calcium concentration since bioluminescence of corals arises in response to various stimuli [24]. The use of CBP as a substrate instead of free coelenterazine increases the efficiency of *in vitro* light emission reaction several times [49] that may be due to the formation of the complex of CBP and Renilla luciferase [50]. It should be pointed out that the efficiency of bioluminescent reaction catalyzing Metridia luciferase also increased with CBP as compared to free coelenterazine [51] even though CBP was isolated from the unrelated organism. It is still unknown how the control of coelenterazine permeability and the neural

control of light response are settled in other animals using coelenterazine as a substrate of bioluminescent reaction. Hence, we can reasonably suggest that the luminous bolus secreted by copepods may represent a complex consisting of several proteins, which may perform various functions such as stabilization of catalytic subunit and substrate delivery to luciferase. Thus, the properties reported in this and similar studies (requirements in detergent and high salt concentration, cooperativity with regard to substrate etc.) may be attributed only to the recombinant luciferases of copepod.

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Figure legends

Figure 1. (A) Protein alignment of *Gaussia princeps* luciferase (GpLuc, GenBank No. AAG54095) and isoforms of *Metridia longa* luciferase (MLuc2, APQ47582, MLuc7, AJC98141). The alignment was produced by ClustalW and manually adjusted using nucleotide sequence information. Red letters indicate the positions in which the amino acid residues are identical, blue letters represent similar residues, and black letters show nonidentical residues. Gaps are shown by dashes. Proposed secretion signals are in gray box. Two most similar motifs within nonidentical repeats are marked by yellow, conservative Cys residues are highlighted in green. The putative duplicated domains in GpLuc luciferase are marked by the arrows (Repeats 1 & 2). The amino acid residue shadowed with cyan indicates the position corresponding to the truncating of high-active ML164M5 mutants of MLuc164 isoform [14]. Variable *N*-terminal sequence between signal peptide and the most conservative part of luciferase sequence (Cons. seq) is underlined in blue. (B) Sequence alignment of the putative duplicated domains in GpLuc. (C) Comparison of amino acid sequences of conservative parts of luciferases.

Figure 2. (A) SDS-PAGE analysis of GpLuc purification from culture medium of insect cells. Lanes: 1, protein standards (Bio-Rad); 2, ammonium sulfate precipitate (40–65%, w/v); 3, sample pooled after chromatography on HisTrap column; 4, flow-through fraction after the second chromatography on HisTrap column; 5, sample after gel filtration on Superdex 75 column; 6, semi-native gel electrophoresis of GpLuc sample after gel filtration on Superdex 75 column. The sample for semi-native gel electrophoresis was prepared without DTT (or β-mercaptoethanol) addition and heating. 12.5% polyacrylamide gels were stained with Coomassie blue. (B) Gel filtration of GpLuc on Superdex 75 column. Column was equilibrated with the buffer 150 mM NaCl, 1 mM EDTA, 5% glycerol, 0.02% NP-40, 20 mM Tris-HCl pH 7.5.

Figure 3. Bioluminescent properties of GpLuc produced in insect cells. (A,B) Dependence of light intensity on temperature and pH, respectively; (C) Effect of NaCl (•) and KCl (Δ) on light intensity; (D) Residual bioluminescence activity after 0.5-h (•), 1-h (○), and 2-h (▲) exposure at different temperatures. GpLuc at concentration of 0.5 μM was in ML buffer with 0.02% NP-40. The bioluminescent activity was measured after 5-min sample cooling. All measurements were carried out in triplicate.

Figure 4. Bioluminescence spectrum of GpLuc at different pH (measurements were carried out in 20 mM Bis-Tris-Propane buffer with 1.0 M NaCl) (A) and log-log plot of dependence of its light intensity on concentration in the range 0.46 fM -0.46 nM (B). Coelenterazine concentration (2 μM) always significantly exceeded that of luciferase. Each point on the plots is the average of three measurements. rlu, initial intensity in relative light units.

Figure 5. Thermally induced unfolding of GpLuc with 0.15 (○) or 1.0 (●) M NaCl monitored by Trp fluorescence (λ_{ex} = 295 nm, λ_{em} = 330 nm). Data are the mean of three independent experiments.

Figure 6. Dependence of initial light intensity of GpLuc on coelenterazine concentration. Insert: Hill plot of GpLuc. Hill coefficient was calculated from the curve slope (y/x). GpLuc concentration was 0.1 nM. Data are the mean of three independent experiments.

Figure 7. Kinetic properties of GpLuc. (A) Log-log plot of stopped-flow records of bioluminescent signals at different luciferase/coelenterazine ratios. Measurements were performed with the use of Applied Photophysics SX20 stopped-flow machine at 18 ^{o}C. Sampling interval was 0.25 ms. Luciferase concentration was 50 nM. (B) Dependence of k_{rise} on coelenterazine concentration.

Table 1. Kinetic properties of GpLuc depending on coelenterazine concentration

Luciferase/substrate ratio	Initial light intensity, rlu	k_{rise}, s^{-1}	$k_{d1,}$ s^{-1}	$k_{d2,}$ s^{-1}
1:1	0.007	19.7 ± 0.2	0.015 ± 0.001	
1:5	0.010	21.6 ± 0.1	0.011 ± 0.001	
1:10	0.016	22.8 ± 0.2	0.014 ± 0.001	
1:15	0.04	25.9 ± 0.3	0.018 ± 0.001	
1:100	1.64	60.0 ± 0.5	0.310 ± 0.003	0.046 ± 0.002
1:200	4.5	89.7 ± 0.5	0.641 ± 0.002	0.080 ± 0.002
1:1000	4.37	147.8 ± 1.4	1.327 ± 0.026	0.169 ± 0.018
1:2000	4.23	150.8 ± 2.0	0.802 ± 0.005	0.059 ± 0.001
1:10000	3.17	195.0 ± 3.8	1.400 ± 0.006	0.015 ±0 .001

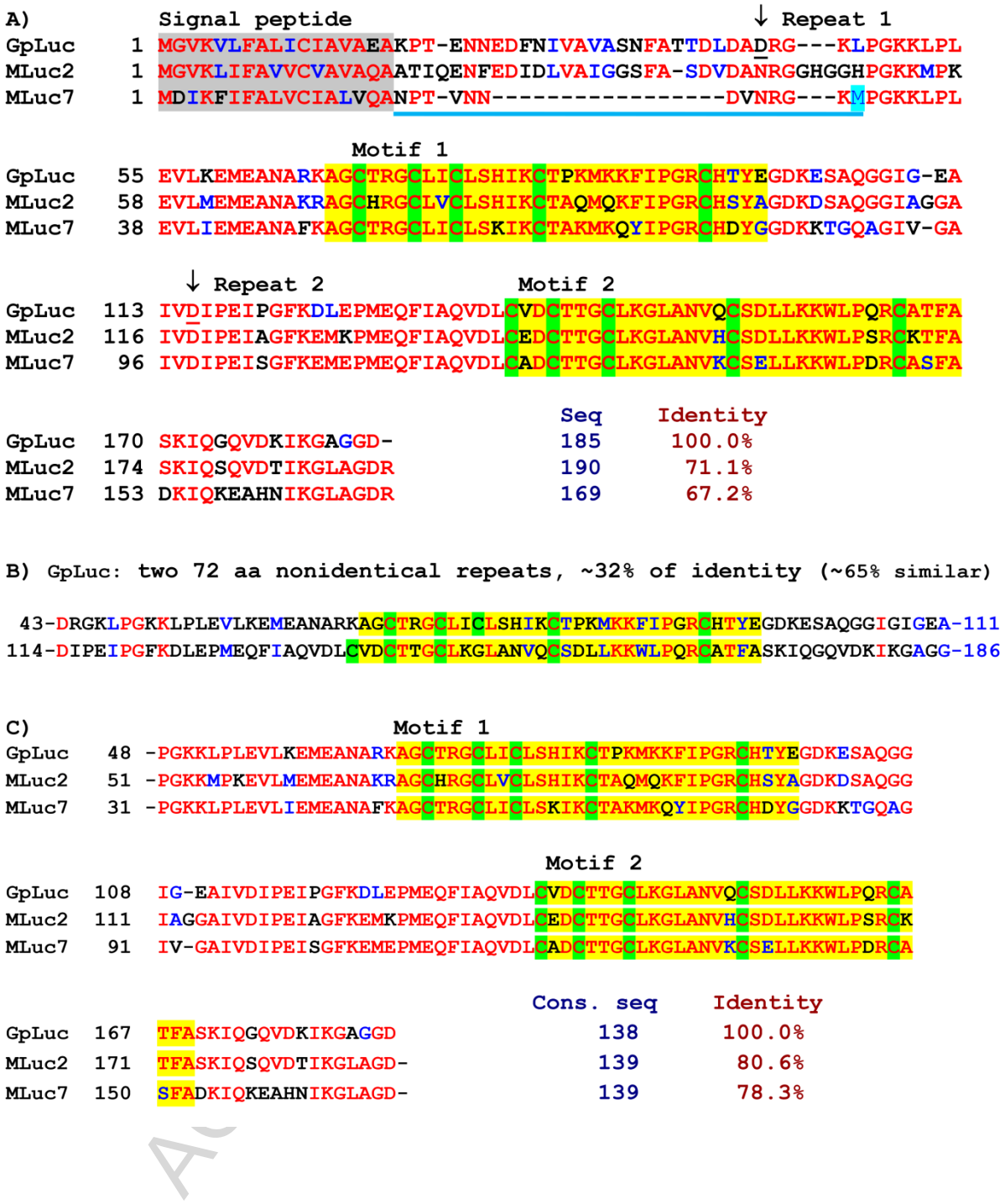


Fig. 1

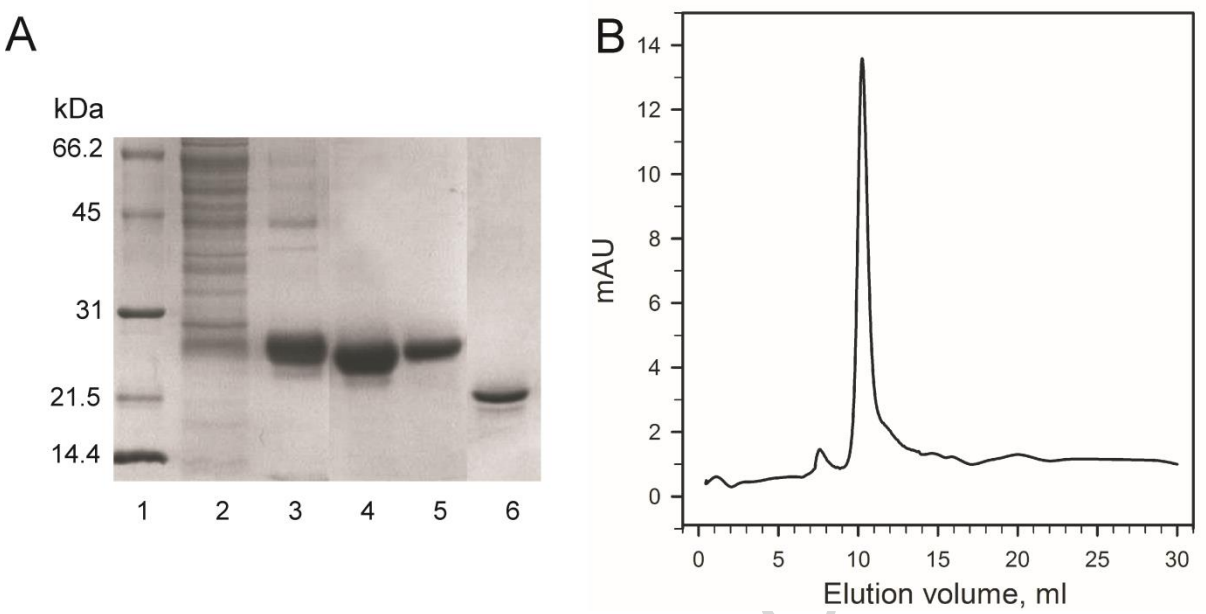


Fig. 2

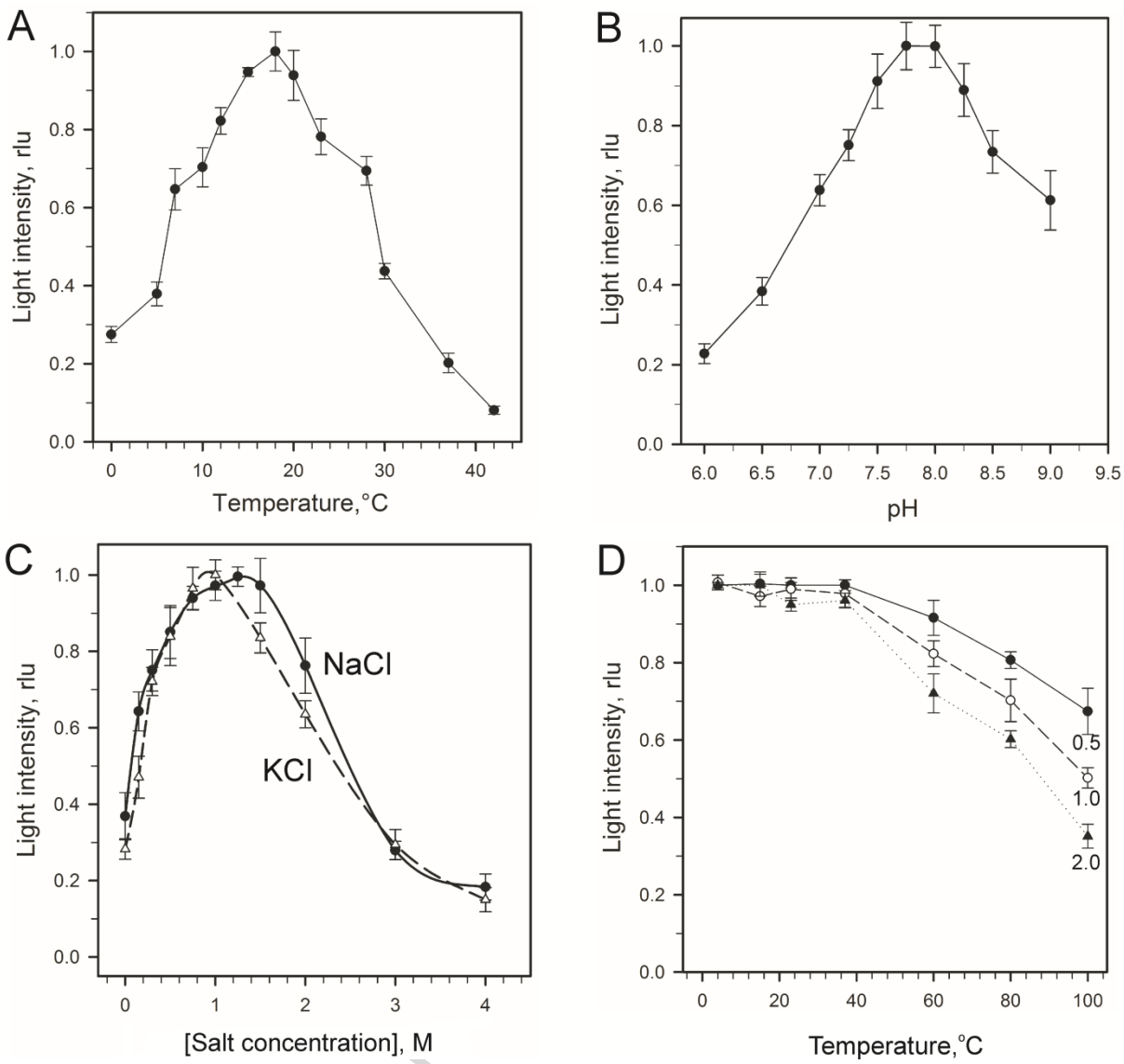


Fig. 3

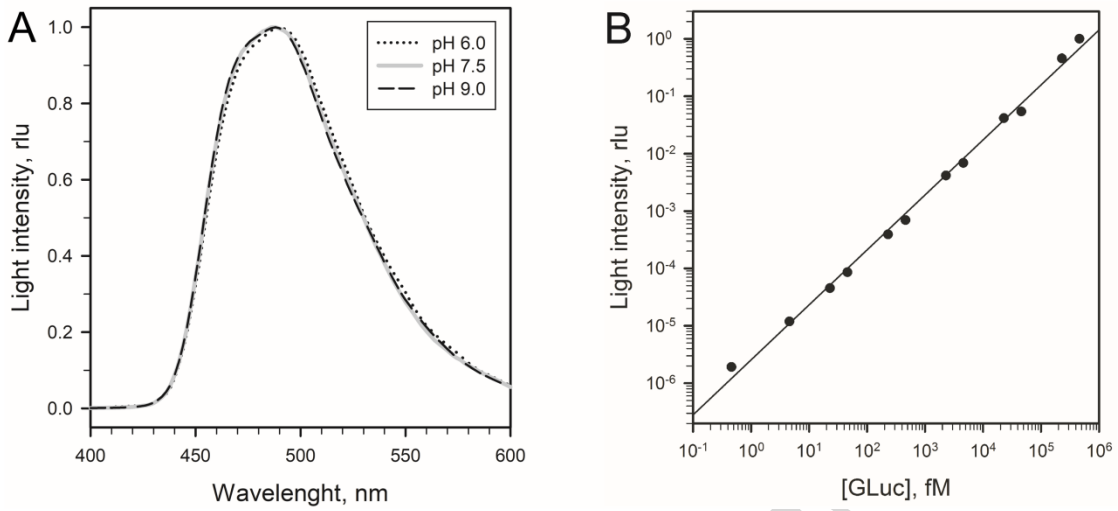


Fig. 4

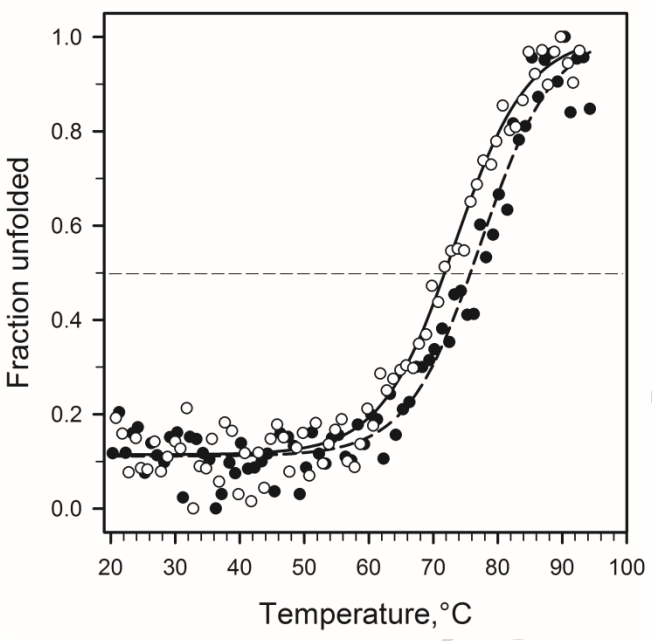


Fig. 5

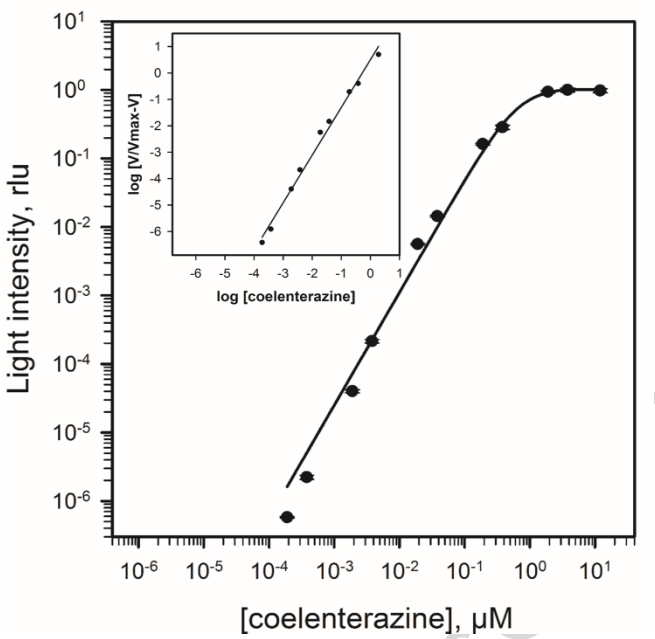


Fig. 6

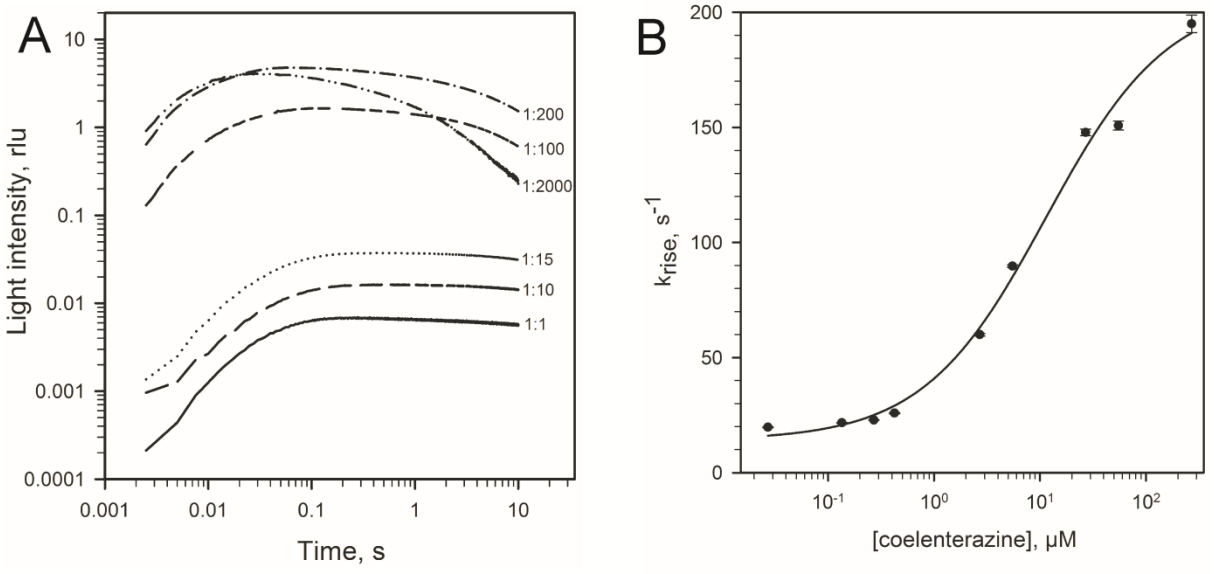
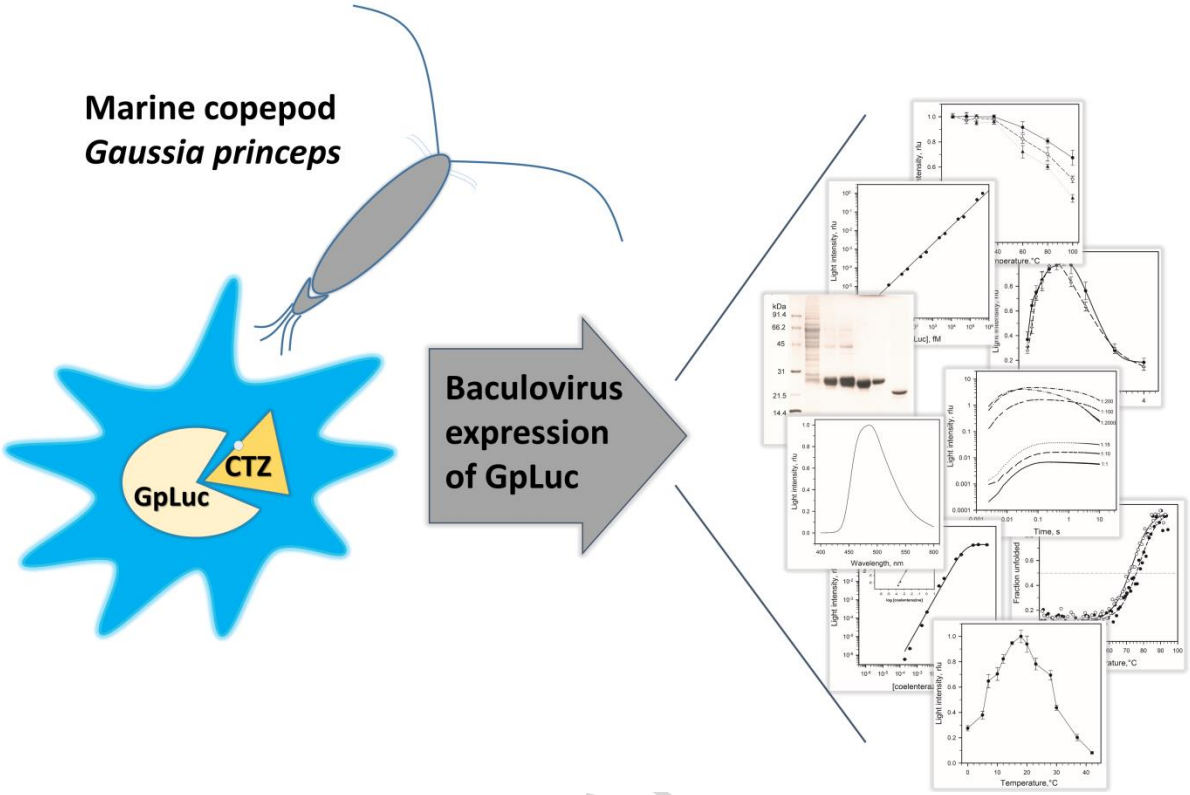


Fig. 7



Graphical abstract

Highlights

- We report properties of proper folded Gaussia luciferase produced in insect cells.
- Luciferase displays features characteristic of halophilic enzymes.
- Gaussia luciferase shows positive cooperativity with coelenterazine.
- We attribute this effect to the so-called kinetic cooperativity.